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TITLE: Modulation of Invading and Resident Inflammatory Cell Activation as a Novel Way to Mitigate Spinal Cord Injury-Associated Neuropathic Pain

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14. ABSTRACT

Experiments completed during the project period were designed to test the hypothesis that the non-psychoactive cannabinoid cannabidiol (CBD) would attenuate spinal cord injury neuropathic pain (SCI-NP) and associated inflammatory markers, and that these protective effects would extend to exacerbating effects of morphine or alcohol exposure on SCI-NP. Our findings from Year 1 demonstrated that CBD treatment attenuates the development of SCI-NP but did not lead to an improvement in locomotor or bladder function. Unlike our original hypothesis, CBD did not have profound effects on microglial activation or on the overall expression of microglial markers, especially those that may promote an anti-inflammatory phenotype. Instead, experiments point to a robust effect of CBD on markers of T cell activation and migration, and a decrease in infiltrating T cells into the injured cord. In Year 2 we determined that morphine exacerbated locomotor and bladder function and that these effects were not counteracted by CBD treatment, although again CBD treatment decreased SCI-NP and T cell infiltration. Lastly in Year 3, we determined that ethanol exposure led to circulating inflammatory markers in the blood and that select inflammatory plasma markers were reduced following CBD treatment.

15. SUBJECT TERMS

Spinal cord injury, neuropathic pain, cannabidiol, inflammation microglia, T cells

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INTRODUCTION

Cannabidiol (CBD) displays a non-psychoactive pharmacological profile as it does not activate CB1 receptors. There is a wealth of safety data already available for CBD from human studies of other disease endpoints, as well as evidence that it is an effective anti-neuropathic agent as a component of the medication Sativex, a buccal spray consisting of THC+CBD approved in the European Union and Canada. Lastly, intense interest already shown by the FDA in the determination of the full range of therapeutic benefits CBD may possess. Experiments completed during the project period were designed to test the hypothesis that the non-psychoactive cannabinoid cannabidiol (CBD) would attenuate spinal cord injury neuropathic pain (SCI-NP) and associated inflammatory markers, and that these protective effects would extend to exacerbating effects of morphine or alcohol exposure on SCI-NP. In **Aim 1 we determined** the effect of CBD on the development and maintenance of spinal cord injury neuropathic pain (SCI-NP) and associated inflammatory markers, including1) the M1 microglial phenotype, and 2) IL-17 producing T (Th17) cells. In **Aim 2** we determined the effect of opioid exposure alone and in combination with SCI on the same inflammatory and neuropathic endpoints, as well as the ameliorative effects of CBD. In **Aim 3** will determine the effect of chronic alcohol consumption alone and in combination with SCI on the same inflammatory and neuropathic endpoints, as well as the ameliorative effects of CBD. Overall our data support the idea that CBD possesses neuroprotective and anti-neuropathic potential that may in large part be due to its ability to suppress pathological T cell activity following insult.

KEYWORDS

Cannabidiol, cannabinoid, delta-9-tetrahydrocannabinol, inflammation, spinal cord injury, neuropathic pain, allodynia, microglia, microglial activation, T cell, infiltration, pro-inflammatory phenotype, anti-inflammatory phenotype, locomotor function, bladder function, morphine, ethanol, alcohol

Accomplishments

Major goals of the project

CY14 Goals

- ❑ Determine effects of CBD treatment on the development of SCI-NP and associated inflammatory markers: **100% complete**
- ❑ Determine the time course of glial and immune cell expression and phenotype during SCI-NP and modulation by CBD: **100% complete**

CY15 Goals

- ❑ Quantify markers following opioid exposure: **100% complete**
- ❑ Investigate combined opioid/SCI effects on inflammation and neuropathic pain: **100% complete**
- ❑ Test non-psychoactive cannabidiol on pain/inflammatory outcomes: **100% complete**

CY16 Goals

- ❑ Quantify markers following alcohol consumption: **100% complete**
- ❑ Investigate combined alcohol/SCI effects on inflammation and neuropathic pain: **100% complete**
- ❑ Test non-psychoactive cannabidiol on pain/inflammatory outcomes: ***50% complete***

Accomplishments under these goals

AIM 1.

Key findings of experiments conducted under specific aim 1 are detailed in the following manuscript under final revision for publication in *Cellular Immunology*.

The non-psychoactive phytocannabinoid cannabidiol (CBD) attenuates pro-inflammatory mediators, T cell infiltration, and thermal sensitivity following spinal cord injury in mice

Running title: Cannabidiol effects in mouse SCI model

Table of Contents title: Cannabidiol attenuates pain and inflammation following spinal cord injury

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Abstract

We evaluated the effects of the non-psychoactive cannabinoid cannabidiol (CBD) on the inflammatory response and recovery of function following spinal cord injury (SCI). Female C57Bl/5 mice were exposed to spinal cord contusion injury (T9-10) and received vehicle or CBD (1.5 mg/kg IP) injections for 10 weeks following injury. The effect of SCI and CBD treatment on inflammation was assessed via microarray, qRT-PCR and flow cytometry. Locomotor and bladder function and changes in thermal and mechanical hind paw sensitivity were also evaluated. There was a significant decrease in pro-inflammatory cytokines and chemokines associated with T-cell differentiation and invasion in the SCI-CBD group as well as a decrease in T cell invasion into the injured cord. A higher percentage of SCI mice in the vehicle-treated group (SCI-VEH) went on to develop moderate to severe (0 – 66% baseline thermal threshold) thermal sensitivity as compared with CBD-treated (SCI-CBD) mice. CBD did not affect recovery of locomotor or bladder function following SCI. Taken together, CBD treatment attenuated the development of thermal sensitivity following spinal cord injury and this effect may be related to protection against pathological T-cell invasion.

Keywords: Spinal cord injury, Neuropathic pain, Cannabidiol, Inflammation, T cell

Abbreviations

BMS - Basso Mouse Scale

CBD – Cannabidiol

SCI – spinal cord injury

SCI-NP – spinal cord injury neuropathic pain

TLR – Toll like receptor

THC - delta9-tetrahydracannabinol

Introduction

Damage to the spinal cord following trauma can lead to motor deficits, changes in sensory sensitivity, and autonomic dysfunction. These deficits result not only from primary insult but also a cascade of reactive changes known as secondary injury. Inflammatory responses such as chemokine and cytokine secretion and immune cell infiltration and/or activation contribute significantly to the development of spinal cord injury as well as to spinal cord injury-associated neuropathic pain (SCI-NP)[1-3]. For example, the incidence of neuropathic pain after spinal cord injury is estimated up to 70% and significantly impacts the patient's life quality[4, 5]. Inflammatory responses, including the generation of free radicals, proinflammatory cytokines, and chemokines, white blood cell invasion, and activation of resident inflammatory cells, are major components of secondary injury. CXCL-1 and CCL-2 are proinflammatory and can function as chemoattractants for neutrophils and monocytes, respectively, while CXCL9 and CXCL11 may function as Tcell attractants. Recently IL-17-producing T cells have been recognized as contributing to the exacerbation of central nervous system (CNS) injury. Toll-like receptor 2 (TLR2) and TLR4 have also been proposed to function in the regulation of proinflammatory cytokines following spinal cord injury[6]. Interference with the pathophysiological changes that occur during secondary injury offers the opportunity to inhibit the progression of damage that usually develops.

Currently therapeutic options available for treatment of spinal cord injury are extremely limited. The current mainstay of medical therapy for acute injury is high-dose methylprednisolone. However, there is considerable debate as to whether the adverse effects of high-dose steroids outweigh the potential benefits of their use [7-9]. Several of the chemical constituents of Cannabis sativa (aka marijuana) have been shown to possess potent anti-inflammatory and neuroprotective properties, and Cannabis has now been legalized in for medical use in twenty-nine states, with alleviation of symptoms relating to autoimmune and chronic pain disorders as top indications. The phytocannabinoids delta9-tetrahydracannabinol (THC) and cannabidiol (CBD) are the two most abundant and well-studied active ingredients in Cannabis. CBD as a monotherapy is currently in dozens of clinical trials for a wide range of indications from schizophrenia to Crohn's disease but has yet to be tested in clinical trials for the treatment of spinal cord injury or chronic inflammatory or neuropathic pain. CBD was shown to improve locomotor function in a rat model of cryogenic

spinal cord injury[10], and we have recently reported that CBD prevents the development of sensory sensitivity in a mouse model of chemotherapy-induced neuropathic pain. Others have shown anti-neuropathic effects of CBD in a rodent model of diabetic neuropathy and chronic nerve constriction [11-14].

CBD enjoys a rich poly-pharmacology with actions on several substrates within the body relevant to inflammation and pain transmission. While its binding affinity for cannabinoid CB1 and CB2 receptors is negligible, it acts as a direct agonist for serotonin 5-HT1A receptors as well as TRPV1 and glycine channels and is an indirect agonist at adenosine receptors[15-18]. These receptor-mediated actions and possible non-receptor mediated effects can attenuate several pro-inflammatory cellular events such as generation of reactive oxygen and nitrogen species, chemokine and cytokine release, microglial and astrocytic activation, and T cell proliferation[19, 20]. Taken together, these data suggest that CBD will be effective for the treatment of secondary insults following spinal cord injury such as those that are involved in the development of SCI-NP. We hypothesized that treatment with CBD would attenuate sensory alterations and decrease inflammation in the spinal cord of injured mice. To test this hypothesis, we used a spinal cord contusion mouse model to induce injury and treated mice with vehicle or CBD (1.5 mg/kg, IP) intermittently for 10 weeks. Preliminary results from our laboratory showed that across a wide range of doses (1.5 – 20.0 mg/kg) and time courses (intermittent versus daily), the present dosing regimen showed optimal results. The effects of CBD treatment on a range of molecular markers of and inflammation as well as motor and bladder recovery, sensory sensitivity, and were determined. qRT-PCR and microarray analyses were conducted at 48 post-injury and flow cytometry analyses were conducted 2 weeks post-injury based on our previous observations with cannabinoid CB2 agonist effects on inflammation following spinal cord injury[21]. Motor, bladder, and sensory function were measured up to 10 weeks post injury.

Materials and Methods

Animal model of spinal cord injury.

Subjects. Seven to 9 week old female C57BL/6 mice weighing 16–21 g (Taconic, Hudson, NY) were used. All procedures, interventions, and animal care were done in accordance with the protocol approved by the Temple University Institutional Animal Care and Use Committee, following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animals were housed for 1 week prior to surgical intervention for acclimation and observation. A light/dark cycle of 12 h was maintained, and the mice were allowed free access to food and water including hydrogel at all times.

Surgical procedures. The mice were weighed and anesthetized via IP injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). Once under anesthesia, the back hair was clipped and protective eye gel was applied. Body temperature was maintained at 37±0.5°C during the procedure and recovery period with a heating pad and lamp. The ribs were used to localize the T8–T10 laminae. Using a combination of sharp and blunt dissection, the paraspinal musculature was dissected free from the laminae between T8 and T10. The laminectomies were performed at the T8 and T9 levels and adequate length of spinal cord was exposed. The mice were then transferred to the Infinite Horizons (IH) impactor device (PSI Inc., Lexington, KY), where they were suspended via modified Adson forceps clamped to the lateral aspect of the vertebra above and below the level of the laminectomy. The impactor tip was positioned directly above the exposed dura, and then raised to a height of 3 mm. The device was set to deliver a 60-kdyn force to the spinal cord. The actual force, displacement, velocity, and injury time was recorded. The spinal musculature and skin was closed with clips.

Postoperative care and CBD treatment. After contusion surgery, the mice were placed in a recovery cage under a heating lamp until they were well recovered from anesthesia. All cages were kept on a heating pad on the first postoperative night. The mice were also given subcutaneous injections of fluid (0.9% NSS; 0.5 mL) and antibiotics (enrofloxacin, 2.5 mg/kg) once daily for the first 3 postoperative days. The mice had their bladders emptied twice daily via the Credé maneuver until recovery of autonomic function.

Cannabidiol (CBD) was dissolved with a 1:1:18 ratio of anhydrous ethanol, cremophor, and 0.9% saline. The mice were randomly divided into vehicle-treated (SCI-VEH; n=7), CBD-treated (SCI-CBD; 1.5 mg/kg CBD; n=8), and sham-treated (SHAM; n=10/group) groups. The SCI-VEH and SCI-CBD-treated mice were given injections 1 hr and 24 hr post injury, and on day 3 and then twice a week by intraperitoneal injection (IP) after surgery, until euthanasia. Mice used for behavioral analyses were in study for 10 weeks post-injury. Additional mice were treated as above for RT-PCR analyses, and were euthanized 48 hr post injury (n=6/group). Lastly, a third set of mice were treated as above and used for flow cytometry (n=6/group) and were euthanized 2 weeks post-injury. The investigators were blinded to treatment during all behavioral scoring.

RT^2 Profiler PCR array. Whole spinal cords were harvested 48 hours after surgery. Three spinal cords were pooled together and homogenized. Homogenized spinal cord tissue underwent mRNA extraction and reverse transcription. Mouse cytokines and chemokines RT^2 Profiler PCR array (Qiagen, Valencia, CA) was performed to detect gene expression. Briefly, 20 µL of cDNA was diluted with 91 µL of RNase free H_2O. Then 102 µL of diluted cDNA was mixed with 1350 µL of 2×SYBR green-containing PCR master mix and 1248 µL of RNase free H_2O to prepare 2700 µL of experimental mixture. Twenty five µL of the mixture was loaded in each well of a 96-well plate pre-coated with primers for different genes. The PCR array was performed using the StepOnePlus Real-Time PCR System (AB Applied Biosystems). The cycling conditions were 15 sec at 95°C, and 1 min at 60°C for 40 cycles, followed by a melting point determination of dissociation curves. Cycle threshold values were determined by automated threshold analysis, and the results were standardized based on 5 housekeeping genes, including glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*), β_2 microglobulin (*B2m*), glucuronidase beta (*Gusβ*), heat-shock protein 90 alpha (*Hsp90sb1*), and *β-actin*.

Quantitative Real time PCR. Whole spinal cord tissue was homogenized in Trizol reagent and underwent mRNA extraction, reverse transcription and qRT-PCR to detect gene expression. The expression of *Il23a(p19), IL23R, IFNγ, CXCL-9, CXCL-11, Nos2, TLR4, TNFα, IL-6, IL-10, and Arg1,* were detected by SYBR Green-based qRT-PCR as previously described[22]. Real-time PCR was performed using StepOnePlus Real-Time PCR System (AB Applied Biosystems). The following primers were used: *Il23a*(p19) sense, 5'-TGCTGGATTGCAGAGCAGTAA-3' and antisense, 5'-ATGCAGAGATTCCGAGAGA-3'; ′; IL23R: sense 5′-ACATTGGACTTTTGTCGGGAA-3′ and antisense 5′-AAAATCGGCAACATG-3′; *Ifnγ* sense, 5’AGCTCATCCGAG and antisense 5’-GCTTCCTGAGGCTGGATACC-3’; CXCL-9: sense 5′-CAAAATTTCATCACGCCCTT and antisense 5′-CCAGACAGCTGTTGTGCATT-3′; CXCL-11: sense 5′-GGGCGCTGTCTTTGCATC and antisense 5′-AAGCTTTCTCGATCTCTGCCAT-3; *Nos2* sense, 5′-CGCAGCTGGGCTGTACAA-3′ and antisense, 5′-TGATGTTTGCTTCGGACATCA-3′; *Il10* sense, 5′-CCTGGTAGAAGTGATGCCCC-3′ and antisense, 5′-TCCTTGATTTCTGGGCCATG-3′; *TLR4* sense 5’GGACCTTACCGGGCAGAAG-3’ and antisense 5’-ACCCCTGGAAAGGAAGGTGT-3’; *Arg1* sense, 5′-GGAAGACAGCAGAGGAGGTG-3′ and antisense 5′-TATGGTTACCCTCCCGTTGA-3′; □*-actin* sense, 5'-TCCACCACCACAGCTGAGAGG-3' and antisense, 5'-CAGCTTCTCTTTGATGTCACG-3'. The expression level of each gene is indicated by the number of cycles needed for the cDNA amplification to reach a threshold. The amount of DNA is calculated from the number of cycles by using standard curves and the results were normalized to □*-actin*.

Flow cytometry. Spinal cord injury mice were treated with Vehicle or CBD for 2 weeks. The mice were anesthetized with 20 µl of mix of ketamine HCl and xylazine and perfused through the left cardiac ventricle with 30 ml of HBSS containing 2mM EDTA. The tissue was homogenized by passing through 18-gauge needle repeatedly after spinal cords and spleens were isolated from animals. Spinal cord tissue and spleen was digested in 5 ml HBSS containing 0.5 mg/ml DNAse I and 0.25 mg/ml Liberase for 45 min at 37°C with shaking, followed by blocking solution (10% FCS, 10 mM EDTA in HBSS). The spinal cord tissue was pelleted and resuspended in 10 ml of 30% isotonic Percoll (diluted with 10x HBSS and distilled water), underlaid with 5 ml of 70% isotonic Percoll. Mononuclear cells were isolated from the 30/70 interphase after gradient centrifugation. Cells were washed with RPMI 1640 medium and counted. FACS analysis was performed to detect CD45+ and CD11b+, and CD4+ cells. Splenocytes were prepared by eliminating erythrocytes with red blood cell lysis buffer according to the manufacturer's protocol (eBioscience). Then splenocytes were counted and underwent FACS analysis to detect CD11b+ cells and CD3+ cells.

Reagents. APC-conjugated anti-mouse CD45, PE-conjugated anti-mouse CD11b, and FITC-conjugated anti-mouse CD3 were purchased from BD PharMingen (San Diego, CA). Trizol reagent was purchased from Invitrogen Corporation (Carlsbad, CA). Percoll was purchased from GE Healthcare (Uppsala, Sweden). Red blood cell lysis buffer was purchased from eBioscience (San Diego, CA).

Motor function evaluation. The mice were evaluated for motor function recovery using the 9-point Basso Mouse Scale (BMS), an open-field assessment of locomotion. Each mouse was evaluated on postoperative days 1, 3, 7 days and once weekly thereafter for 10 weeks.

Autonomic function evaluation. All mice had autonomic impairment with urine retention following SCI. To relieve their bladders and to assess autonomic function recovery, urine was expressed twice daily via suprapubic pressure (Credé maneuver), and urine mass was determined. The mice were considered to have recovered autonomic function once the total urine mass expressed was less than 500 mg/day for 3 consecutive days.

Thermal sensitivity test. Thermal sensitivity of the right hind paw was assessed using a standard rodent thermal algesia meter (Hargreaves Apparatus, 37370, Ugo Basile). Mice were habituated to the compartments for 15 minutes. The infrared source (intensity 25) was then placed under the glass floor and aimed at the center plantar surface of the right hind paw. When the mouse withdrew the hind paw the latency time is automatically recorded. Each mouse was tested 3 times with an inter-test interval of 3 minutes, and average withdrawal latency is determined. If there is no observed response in 22.3 second, the trial was ended automatically to avoid tissue damage.The plantar test in right hind paw in mice was performed before surgery and in 4, 6, and 10 week after surgery. The latency to respond for each mouse after injury was compared with individual baseline before surgery to obtain percent baseline response levels. Based on the distribution of thermal sensitivity scores in the vehicle-treated injured mice, response levels were also divided into 2 categories: ***mild***, or thermal sensitivity scores that represented a ≥66 - 100% baseline threshold response, and ***moderate to severe***, or thermal sensitivity scores that represent a 0 – 66% baseline threshold response. Based on our preliminary and present experimental groups, our vehicle-treated SCI mice divide roughly in half into these two categories.

Mechanical allodynia test. Mechanical sensitivity of the right and left hind paws was measured using standard von Frey filaments (Exacta Touch Test). A baseline measure for each paw was taken prior to inducing the spinal cord injury. Mice were placed in confined chambers on top of an elevated metal grid (Bioseb *In Vivo* Research Instruments) and acclimated to the environment for 30 minutes prior to testing. Two weeks post spinal cord injury mechanical

allodynia testing was conducted once a week for 8 weeks. The up down method described in Chapman et al 1994 was used with filaments ranging from 0.16 – 2.0 grams of force[23]. Pressure was applied to the right hind paw with enough force to create a "C" shape and this pressure was applied for 6 seconds for each filament used. The threshold to respond for each hind paw after injury was compared with individual baseline data before surgery to obtain percent baseline response levels. In addition, because several mice showed both decreased and increased mechanical sensitivity time and between left and right paw measurements, percent baseline changes for each mouse at each time point were calculated for their more sensitive paw measurement and for their less sensitive paw measurement.

Statistics analysis. Results are summarized as mean ± SEM. Comparisons between two groups (flow cytometry) were done using Student t test whereas comparisons among multiple groups were performed using mixed-effects linear regression or mixed-effects logistic regression/generalized estimating equation (GEE) analysis with Tukey-Kramer multiple comparison post-test adjustments to take the longitudinal correlation in the data into account. Treatment, time, and their interaction terms were included in the regression models whenever appropriate. P-values less than 0.05 were considered statistically significant. SAS version 9.3 (SAS Institute Inc., Cary, NC) was used for all the data analyses.

Results

Impact results. For the SCI-VEH group, the average kDyn of force measured at time of impact was 63.57 ± 1.90 and the average displacement was 375 ± 29.43 microns. For the SCI-CBD-treated group, the average kDyn of force measured at time of impact was 62.38 ± 1.83 and the average displacement was 363.38 ± 20.54 microns.

SCI-CBD significantly attenuated increased cytokine expression in the injured spinal cord. PCR array analysis of spinal cords harvested 48 hr post-injury in vehicle- and CBD-treated mice (Table 1) was performed to measure changes in gene expression of 84 key genes mediating the inflammatory response. CBD treatment was associated with the down-regulation of several chemokines and interleukins, including Ccl11, Cxcl22, Cxcl9, Xcl1, IL12b, and IL17a, and was associated with increased expression of Ccl1, Ccl12, Ccl20, Ccl24, and IL9 (Table 1). Some gene expression changes were confirmed by RT-PCR and other targets of interest were included (Figure 1). One-way ANOVA with Tukey's multiple comparison post-test adjustments showed that SCI-VEH significantly increased expression of IL-23 ($p=0.03$) and its receptor ($p<0.0001$), INFγ ($p=0.02$), CxCl-9 ($p<0.0001$), CxCl-11 ($p=0.01$), iNOS ($p<0.0001$), TLR-4 ($p<0.0001$), IL-10 ($p<0.0001$), Arg1 ($p<0.0001$), TNFα ($p<0.0001$), and IL-6 ($p<0.0001$)as compared with SHAM (significance indicated by * on figures). SCI-CBD significantly decreased the expression of IL-23 ($p=0.05$) and its receptor ($p=0.007$), CXCL-9 ($p<0.0001$), CXCL-11 ($p=0.001$), and iNOS ($p<0.0001$), and significantly increased the expression of TRL4 ($p=0.001$) as compared with SCI-VEH mice (significance indicated by # on figures). CBD treatment did not statistically significantly change INFγ ($p = 0.07$, Arg-1, IL10 ($p =0.06$) or IL6 expression compared to SCI-VEH.

CBD significantly reduced CD4+ T cell numbers but not macrophage or microglia number in the injured spinal cord. CBD treatment did not significantly decrease macrophage (CD45+high/CD11b+), microglia (CD45+intermediate/CD11b+), or total CD45+ cell populations in the spinal cord compared to vehicle-treated spinal cord-injured mice. CBD treatment did significantly decrease the total number of CD4+ T cells in the injured cord compared with vehicle treatment (Figure 2A). To ensure that the changes in cellular invasion were not the result of a systemic disease, total cell, macrophage, and CD3+ T cell numbers in the spleens of CBD- and vehicle-treated animals were compared. The results showed there was no significant difference in cell population between the two treatment groups (Figure 2B).

CBD did not significantly improve motor or bladder function following spinal cord injury. There was no statistically significant effect of SCI-CBD treatment on BMS score when compared to the SCI-VEH group [$F_{(1,13)}$<1.0, n.s.]. There was an overall main effect of time [$F_{(7, 91)}$ = 136.5, p <0.0001], and no significant interaction [$F_{(7, 91)}$ = 1.1, n.s.] (Figure 3). CBD treatment did not increase the number of mice who recovered bladder function by 4 weeks post-spinal cord injury (70.4% for SCI-VEH, 68.8% for SCI-CBD group).

CBD treatment leads to less severe thermal sensitivity after spinal cord injury. Following injury, median percent thermal threshold scores compared to baseline were approximately 50% by week 10 post-injury in SCI-VEH mice and approximately 70% in SCI-CBD mice. Mixed-effects linear regression showed a significant main effect of treatment [$F_{(2, 22)}$ = 20.75, p<0.0001], but no significant main effect of time [$F_{(2, 44)}$ < 1, n.s.] and no significant interaction between treatment and time [$F_{(4, 44)}$ = 1.17, n.s.]. Tukey's multiple comparison post-tests showed that at Week 4, both SCI-VEH and SCI-CBD mice were significantly more sensitive compared with SHAM mice, at Week 6 only SCI-VEH mice were significantly different from SHAM mice, and at Week 10 both SCI-VEH and SCI-CBD mice showed significantly more sensitivity compared with SHAM mice (Figure 4A). As described in the methods section, because we observe a range of the severity of thermal sensitivity following SCI, we also analyzed the data by generating two sensitivity categories ("mild" = ≥66 - 100% baseline thermal threshold response and "moderate to severe" = 0 – 66% baseline thermal threshold response) and calculating the percentage of animals which fell into each category by treatment group and time point. Approximately 70% of SCI-VEH mice went on to develop moderate to severe thermal sensitivity that progressed in severity between 4 to 10 weeks post-impact (similar to what has been reported previously by Nesic et al 2005, while only 25% of SCI-CBD mice developed moderate to severe thermal sensitivity (Figure 4B) [24]. GEE/mixed-effects logistic regression analysis showed a significant effect of treatment (p=0.01) but no significant effect of time (p=0.85). There were too few observations to analyze the data for a significant interaction. Post-test analysis showed that vehicle treated mice had a significantly higher proportion of "moderate to severe" than the sham injured mice across time (p<0.01). SCI-CBD treated mice were not significantly different from SHAM mice across time.

The T9-10 SCI contusion mouse model was not significantly associated with increased mechanical sensitivity. Unlike what was observed for thermal sensitivity, spinal cord injured mice did not develop significant sensitivity to touch (Figure 5A, data for right paw shown for consistency with thermal sensitivity data). In fact, although a weak trend toward reduced sensitivity in the SCI-VEH and SCI-CBD groups compared with the SHAM group was observed during the first half of the 10 week study, mixed-effects linear regression analysis revealed no statistically significant main effect of treatment [$F_{(2,22)}$ = 0.98, p=0.39], and no statistically significant main effect of time [$F_{(8,175)}$ = 1.03, ns], and no statistically significant interaction between treatment and time [$F_{(16,175)}$ =1.17, ns]. As mentioned in the Methods and depicted in Figure 5A, the data were variable, reflecting both increases and decreases in mechanical sensitivity, sometimes in the same mouse at the same time point between the right and left paws. Therefore, we also analyzed the more sensitive and less sensitive values obtained by mice at each time point Figure 5B). When the more sensitive paw values were averaged from each animal at each time point, it was observed that SCI-VEH and SCI-CBD mice seemed to display higher sensitivity to touch than SHAM mice. Mixed-effects linear regression analysis revealed a marginally significant main effect of treatment [$F_{(2,22)}$ = 2.42, p=0.11], and no statistically significant main effect of time [$F_{(8,175)}$ = 1.05, ns], and no statistically significant interaction between treatment and time [$F_{(16,175)}$ =1.16, ns]. Tukey-Kramer's post-tests showed (marginally) significant differences between CBD and sham-injured groups at week 4 (p=0.08) and between vehicle and sham-injured groups at week 4 (p=0.06), week 6 (p=0.03), and week 8 (p=0.02). When the less sensitive paw values were averaged, it was observed that SCI-VEH and SCI-CBD mice displayed less sensitivity to touch than SHAM mice. Mixed-effects linear regression analysis revealed a statistically significant main effect of treatment [$F_{(2,22)}$ = 3.70,

p=0.04], and no statistically significant main effect of time [$F_{(8,175)}$ = 1.32, ns], and no statistically significant interaction between treatment and time [$F_{(16,175)}$ = 0.78, ns]. Tukey-Kramer's post-tests showed marginally significant differences between SCI-CBD and SHAM groups at week 6 (p=0.09), and marginally significant differences overall among the three groups at week 2 (p=0.03), week 5 (p=0.07), and week 6 (p=0.07).

Discussion

In the present study we demonstrated that following spinal cord contusion injury in mice, CBD treatment was associated with a decrease in several pro-inflammatory cytokines and chemokines associated with secondary injury following damage to the spinal cord and with the development of neuropathic pain. CBD also significantly attenuated the presence of CD4+ T cells, but not CD45+ macrophages or microglia, within the damaged spinal cord. These neuroimmune outcomes were associated with a decrease in thermal sensitivity in these mice. Results also showed that changes in mechanical sensitivity in this mouse model of thoracic contusion injury were variable even between the left and right hind paw of an individual mouse, with increases and decreases in sensitivity observed. There was a trend toward statistical significance for the effect of SCI, regardless of vehicle or CBD treatment, to increase sensitivity, while there was a significant effect of SCI on insensitivity with a trend toward SCI-CBD producing the most mechanical insensitivity. CBD treatment also did not improve motor or bladder function.

As neuroinflammation is associated with SCI and SCI-NP, we determined the effects of CBD treatment on immune markers in SCI mice 48 hr post injury. Microarray analysis (Table 1) showed that CBD downregulated or upregulate by several fold, a multitude of key chemoattractant factors and cytokines. qRT-PCR results similarly showed that CBD decreased IL-23 and its IL23 receptor and reduced expression of CXCL-9 and CXCL-11, IFNγ and iNOS. No significant reductions in TRL4 receptor, Arg-1, and IL-10 expression were observed. Taken together, these results suggest that CBD may attenuate neuroinflammation by suppressing the expression of molecules involved in immune cell communication, activation, migration and cross-talk with neuronal populations.

We and others have demonstrated that resident microglia can be activated chronically after spinal cord injury in both experimental animals and patients [21, 25-27]. Moreover, there is a growing literature implicating spinal microglial activation in the development of neuropathic pain by enhancing the hyperactivity of spinal nociceptive neurons and promoting central sensitization [28-31]. In addition, others have reported in alternate models of neuropathic pain that CBD's anti-neuropathic effects co-occur with a reduction in microglial activation [13, 14, 32]. Some changes in cytokine and chemokine expression following CBD treatment observed in the present study are indicative of alterations in macrophage/microglial activation, such as Il1β, IL12β, and IL23α. However, expression of other common markers of microglial activity remained unchanged with CBD treatment, including the TRL4 receptor, Arg-1, TNFα, IL6, and IL10 expression. This observation is further substantiated by the flow cytometry results showing no significant decrease in microglial and macrophage populations within the injured spinal cord in CBD-treated vs vehicle-treated mice. However it must be noted that while microglial number was not reduced by CBD treatment, this does not preclude a possible effect of CBD on the functionality of these cells following injury.

A major function of activated microglial and macrophages following injury is the recruitment of lymphocytes such as T cells. Indeed in contrast to the microglia and macrophage results, flow cytometry revealed a significant reduction in CD4+ T cells following treatment with CBD. In agreement, a majority of mRNA expression changes observed with CBD in this study implicate alterations in T cell activation and migration, including Xcl-1, Ifn-γ, Ccl-9, Ccl11, CcL-20, Ccl-22, lL-17, IL-12β, IL-23 and its receptor. In addition to microglia and macrophages, T cells have also been strongly implicated in the secondary inflammatory response following spinal cord injury [33-35]. More recently T cells have been suggested to play an essential role in

SCI-NP, although the underlying mechanisms remained understudied [36-38]. Perhaps most relevant to the present results and the preclinical modeling of SCI in general is the reported differential role that microglia versus lymphocytes may play in the modulation of neuropathic pain in male and female models. Of course this is especially important to consider given the heavy reliance on female rodent models for SCI studies. Taken together, these data suggest that CBD may either decrease the release of T-cell chemo-attractant molecules from cells such as microglia, monocytes, and dendritic cells, or act directly on T cells to inhibit activation and/or migration. Again, while we did not see a significant effect of CBD treatment on microglial and macrophage number, we cannot rule out that CBD alters the phenotype of these cells to regulate their functionality. However, there is mounting evidence from elegant cell culture studies that CBD application can directly alter T cell gene expression and phenotype [20, 39].

In our spinal cord contusion mouse model, we observed a range in the degree of thermal sensitivity developed across vehicle-treated SCI subjects, and analysis of mean % baseline thermal threshold scores showed this variability, with a trend toward CBD attenuating the development of thermal sensitivity following SCI. Based on this wide distribution, we also analyzed our data by dividing subjects into 2 categories: mild, or thermal threshold scores that represented a ≥66 - 100% baseline response, and moderate to severe, or thermal threshold scores that represent a 0 – 66% baseline response. As seen in Figure 1B, the percentage of mice that developed moderate to severe thermal sensitivity is similar between vehicle- and CBD-treated SCI at week 4. However, at weeks 6 and 10, approximately 70% of vehicle-treated mice show moderate to severe sensitivity, while only 20% of CBD-treated mice do.

In contrast to effects on thermal sensitivity, we found little evidence of the development of below-level mechanical sensitivity in our model. Specifically, when compared to sham-injured mice, mice exposed to SCI showed an increase in mechanical threshold indicative of a loss of tactile sensation, with a trend toward CBD producing the most mechanical insensitivity. Mechanical allodynia is a common observation in rats following contusion injury of the spinal cord, but a review of literature shows a more complex range of outcomes in mouse models. Even among studies that focus on contusion injury of the thoracic T9-12, resultant effects on tactile sensitivity vary across reports. For example, Hoschouer et al 2009 reported no changes in mechanical sensitivity following contusion injury to the thoracic region, while Chen et al 2012 reported tactile allodynia in their model[40, 41]. Moreover, insensitivity has been reported at early time points and hypersensitivity at later time points, and hypersensitivity to thin filaments but hyposensitivity to thicker filaments[42, 43]. Taken together the presence and direction of tactile changes following spinal contusion injury in mice is variable across laboratories and likely relates to the level and nature of damage to the dorsal column pathway transmitting ascending mechanical touch information.

While CBD treatment was associated with less severe thermal sensitivity, this was not accompanied by improvements in motor and bladder function recovery that we have previously reported with treatment of the cannabinoid CB2 receptor agonist O-1966 [21]. These results emphasize the very complex pathophysiological mechanisms underlying SCI and SCI-NP. Clearly, both motor and sensory pathways are damaged in this model and our data suggest that treatment with CBD preferentially protects from dysregulation of nociceptive systems over attenuation of/ repair to damaged touch or motor pathways.

In conclusion, our data suggest that treatment with CBD can mitigate the development of thermal sensitivity following spinal cord injury, and that these effects may in part due to a suppression of release of cytokines, chemokines, and other signaling molecules involved in T cell activation and recruitment into the spinal cord. In addition, these effects seem largely separate from improvements in motor and bladder function, suggesting that mechanisms involved in the development of neuropathic pain following spinal cord injury are not identical to those that underlie deficits in motor and autonomic function. Further research is necessary in vivo to determine the initial site(s) of action for CBD necessary to attenuate the development of neuropathic pain following spinal cord injury.

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Figure legend

Figure 1. SCI-CBD significantly attenuated increased cytokine expression in the injured spinal cord. RT-PCR 48 hr following injury showed that SCI-VEH mice had increased expression of IL-23, the IL-23 receptor, IFNγ, CxCl-9, CxCl-11, iNOS, TLR-4, IL-10, TNFα, IL-6, and Arg1 as compared with SHAM mice ($p < 0.05$, indicated by asterisks on figure). SCI-CBD significantly reduced expression of the cytokine IL-23p19 subunit, its receptor IL-23r, the chemokines CXCL-9 and CXCL-11, IFNγ, and iNOS and significantly increased TLR-4 expression compared with the SCI-VEH mice ($p < 0.05$ as indicated by pound sign on figure). In contrast, there were no significant effects of SCI-CBD versus SCI-VEH differences in IL-10, TNFα, IL-6 and Arg-1 expression. (n=6/group).

Figure 2. SCI-CBD significantly decreased CD4+ T cell population in the injured spinal cord, but does not significantly alter microglial or macrophage populations. Spinal cords were harvested 2 weeks following SCI-VEH or SCI-CBD treatment. SCI-CBD did not significantly decrease total CD45+ population (Figure 5A top left), microglia (bottom left), and macrophage (top right) population, but did significantly decrease CD4+ T cell population (bottom right). There were no significant differences in total spleen cell, T cell, or macrophage numbers in the spleens of CBD-treated versus vehicle-treated animals (Figure 5B) (n=6/group).

Figure 3. SCI-CBD did not significantly affect motor function following spinal cord injury (Basso mouse scale; BMS). Locomotor recovery was assessed using the BMS. BMS scores for mice in each treatment group at each time point were averaged (±SEM). Mixed-effects linear regression showed no statistically significant effect of SCI-CBD as compared with SCI-VEH.

Figure 4. CBD treatment attenuates the development of thermal sensitivity in the hind paw following spinal cord injury. Mice received sham or T9-10 contusion injury and treated with vehicle (SCI-VEH) or 1.5 mg/kg CBD (SCI-CBD) 1h pre and 24h post injury and twice weekly thereafter for 10 weeks. Thermal sensitivity of the right hindpaw was measured using the plantar test. (A) The percent baseline latency scores for each treatment group (SHAM, n=10; SCI-VEH, n=7; SCI-CBD, n=8) at each time point (4, 6, and 10 weeks post-injury) were averaged (±SEM). Mixed-effects linear regression revealed a significant treatment effect; * represents adjusted $p<0.05$ as compared to sham control using Tukey-Kramer's multiple comparison method. (B) The percentage of mice in each treatment group which displayed moderate to severe changes in thermal sensitivity (thermal sensitivity scores that represent a 0 – 66% baseline response) is depicted. Mixed-effects logistic regression analysis showed a significant overall treatment effect, revealing a statistically significant difference between SHAM and SCI-VEH groups ($p<0.05$, indicated by dotted line).

Figure 5. The T9-10 SCI contusion mouse model was not significantly associated with increased mechanical sensitivity. Mechanical sensitivity of the right and left hind paw was measured using the Von Frey test. The percent baseline sensitivity scores from the right paw for

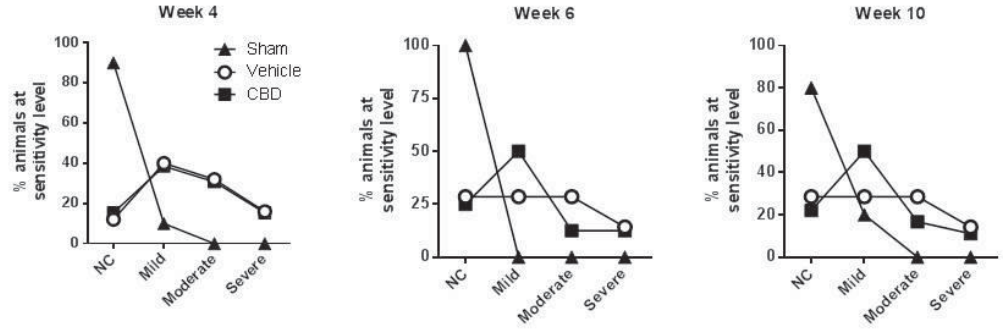
each treatment group at each time point (4, 6, and 10 weeks post-injury) were averaged (±SEM). For the right hindpaw, which was measured for the thermal sensitivity studies, mixed-effects linear regression revealed no overall significant effect of treatment, time or their interaction (Figure 2A). The more sensitive percent baseline scores for each mouse (left vs right paw) were also averaged and statistically compared between groups and across time (Figure 2B), and mixed-effects linear regression revealed a marginally significant overall treatment effect. The less sensitive percent baseline scores for each mouse (left vs right paw) were also averaged and statistically compared between groups and across time (Figure 2C), and mixed-effects linear regression revealed a significant overall treatment effect ($p < 0.04$ indicated by bracket and asterisk) and a marginally significant difference between SHAM and SCI-CBD on Week 6 ($p = 0.09$ indicated by asterisk above data point) with multiple comparison post-test adjustments.

Table 1 Microarray analysis of gene regulation in SCI-VEH- versus SCI-CBD mice 48 hr after spinal cord injury. The genes shown in the table illustrate the expression of 9 genes upregulated by CBD treatment ≥ 4-fold and the expression of 9 genes downregulated by CBD treatment ≥ 4-fold out of 84 key secreted proteins central to the immune response.

Genes upregulated by CBD treatment	Fold difference	Genes down-regulated by CBD treatment	Fold difference
Ccl1	13.4064	Cxcl11	-9.6237
Ccl12	6.2994	Cxcl22	-15.7553
Ccl20	22.2876	Cxcl9	-9.2118
Ccl24	10.2979	Il2	-5.6824
Cxcl13	4.4604	Ifng	-10.152
Il5	6.3942	Il1b	-6.1986
Il9	14.2601	Il17a	-9.4012
Mstn	4.3824	Il12b	-8.4049
Tnfsf10	4.6432	Xcl1	-29.057

Figure 1

A.
Thermal sensitivity - 5 week treatment



B.
Thermal sensitivity - 10 week treatment

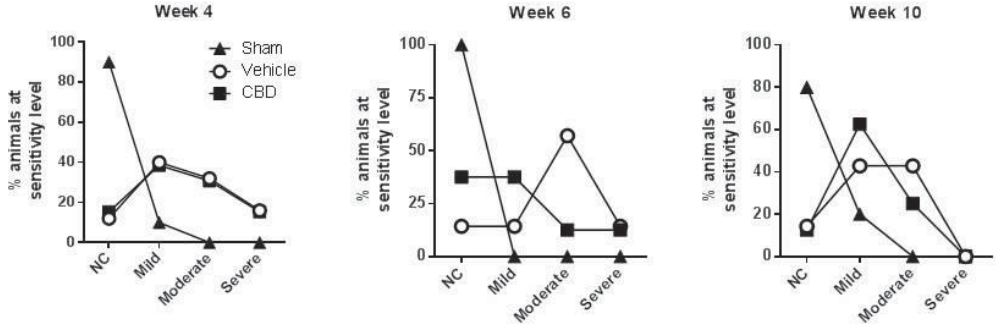


Figure 2

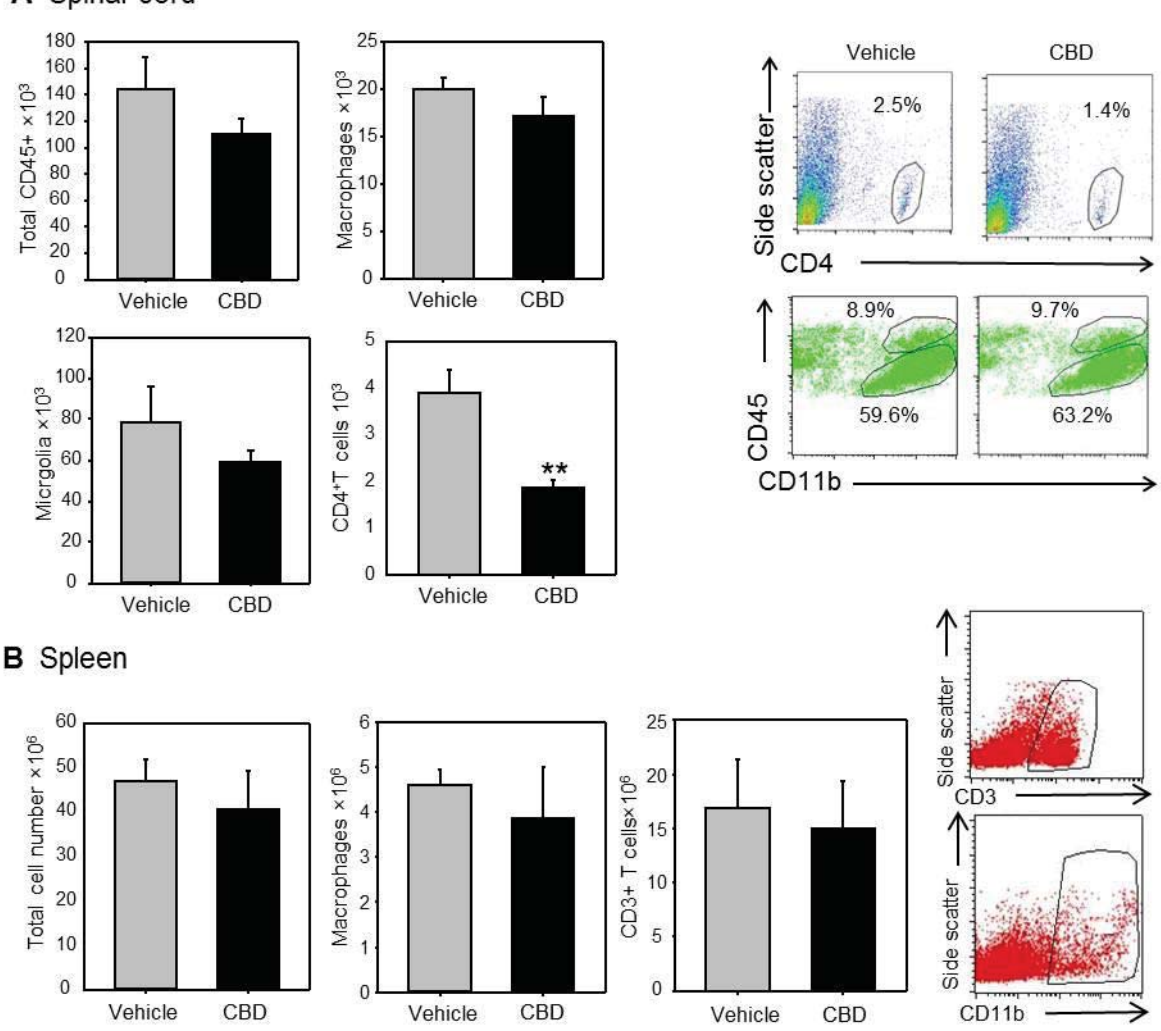


Figure 3

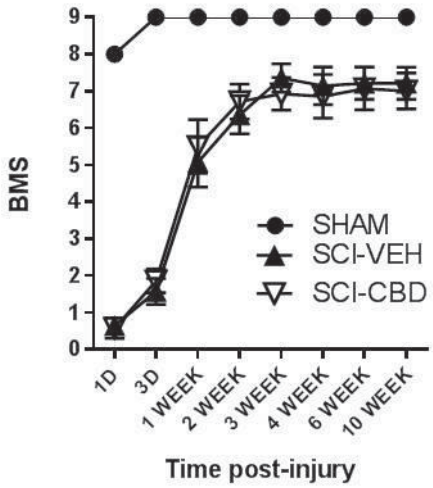


Figure 4

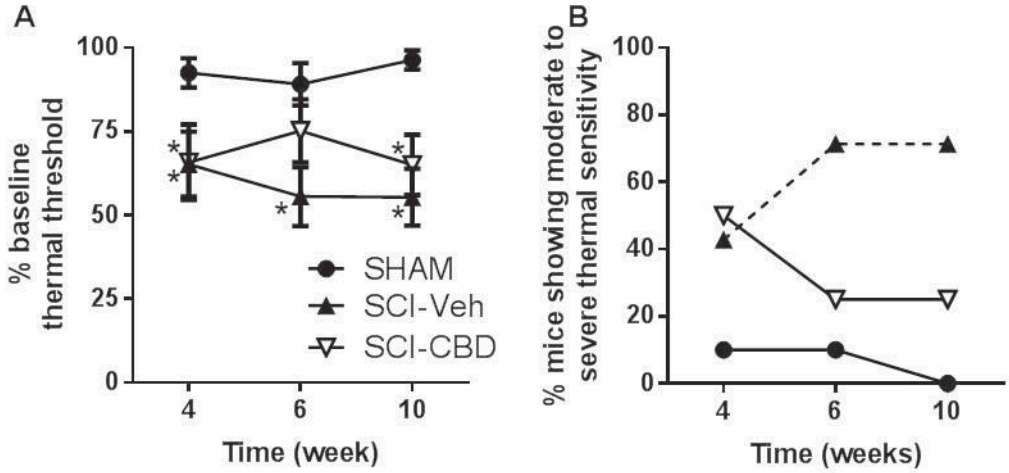
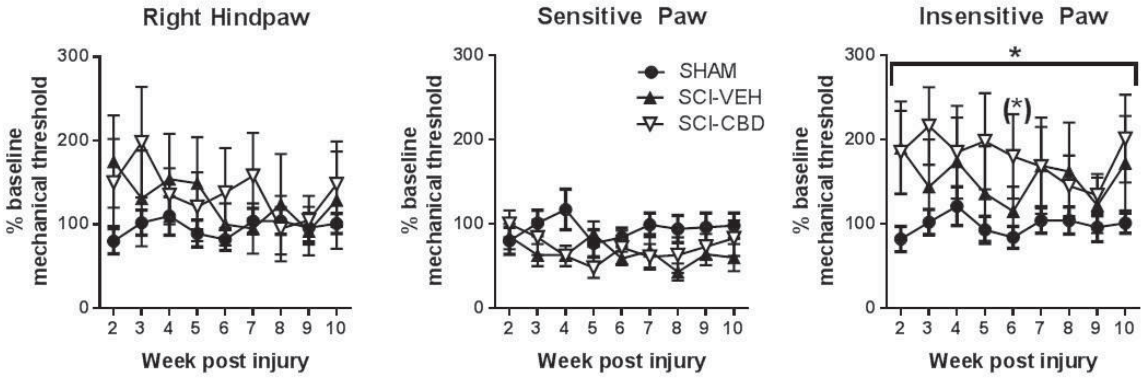


Figure 5



AIM 2.

Key findings of experiments conducted under specific aim 1 are detailed in the following presentation given at the Mid-Atlantic Pharmacology Meeting in October of 2017.

Continuous morphine infusion deteriorates locomotor recovery and enhances chronic neuropathic pain in spinal cord injury mice and effects of CBD treatment

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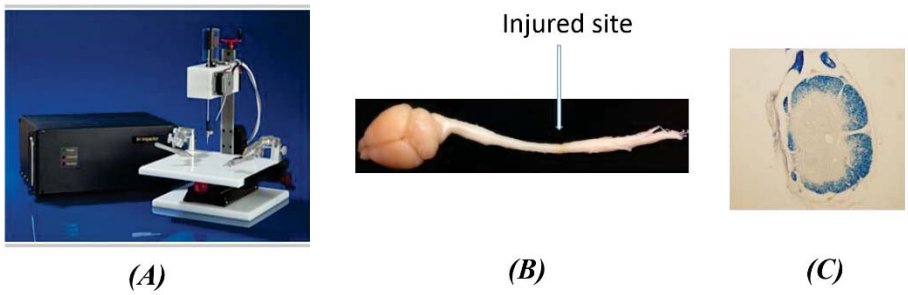
Center for Substance Abuse Research, Lewis Katz School of Medicine, Temple University

Introduction: Morphine, which belongs to opiate class of analgesics, is widely used in the clinic to treat acute and chronic pain. However, morphine not only has high potential for addiction and also could worsen recovery and pain through proinflammatory mechanisms. For example, morphine was proved to activate neuroinflammation by binding to myeloid differentiation protein 2 (MD2), an accessory protein of Toll-like receptor 4(TLR4), which is mainly expressed by macrophage and microglia. Cannabidiol is the non-psychoactive ingredient in Cannabis, and we recently found it can ameliorate thermal sensitivity following spinal cord injury by modulating the immune response. **Aim:** The hypothesis of our research was that using a combined treatment of morphine and CBD would lead to an improvement in recovery compared with morphine alone.

Methods: The surgeries for spinal cord injury or sham were done in female C57/BL6 mice and mini-pumps containing 98.8ul morphine in 2.52mg/100ul, 3.78mg/100ul concentration or control vehicle solution were implanted subcutaneously, infusing morphine 0.5ul per hour, 24 hours continuously for 7 days. A subset of spinal cord injured mice with 3.78mg/100ul mini pumps were given vehicle or CBD treatment 1 hour post injury and every 24 hours for 7 days by intraperitoneal injection. Changes in locomotor and bladder function and hindpaw thermal and tactile sensitivity in injured mice were evaluated.

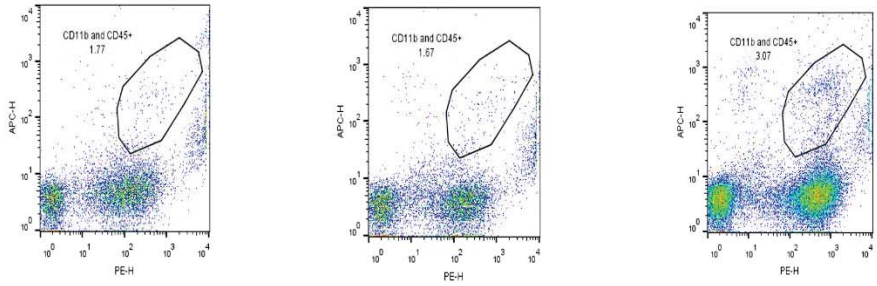
Results:

Figure 1. The model of spinal cord injury in mouse.



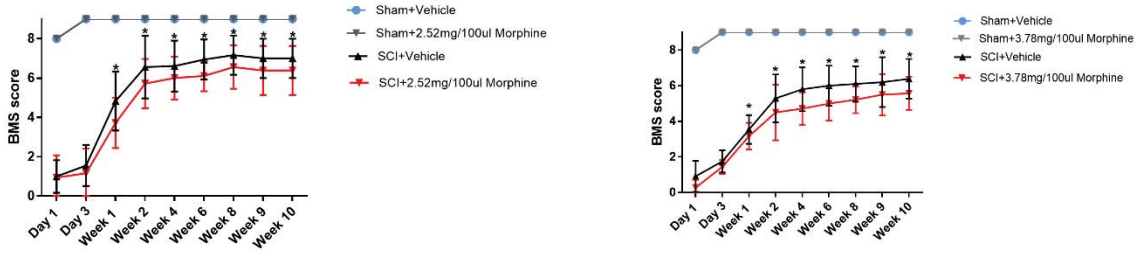
(A) Infinite horizon impactor was used to make spinal cord injury model.(B) The injured spinal cord was dissected 4 weeks after surgery and the arrow showed the injured site. (C) The epicenter section of spinal cord injured tissue was stained with Eriochrome cyanine R and cresyl violet.

***Figure 2*: The macrophage/microglia population increased in sham mice infused with 3.78mg/100ul morphine.**



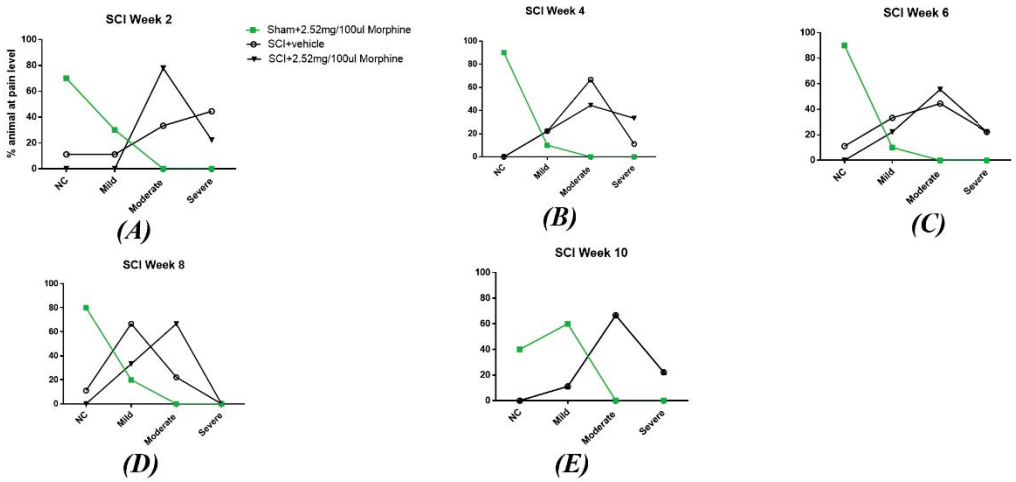
The sham mice of spinal cord injury had surgery without contusion and were implanted for 7 days with a mini-pump (0.5ul/h, 98.8ul) which was filled with saline (n=2), 2.52mg/100ul (n=3) or 3.78mg/100ul (n=3) morphine. The spinal cords of mice in each group were collected after 2 weeks and combined. The immune cells were isolated, stained and run flow.

***Figure 3*. Morphine infusion deteriorated locomotor function in spinal cord injury mice.**



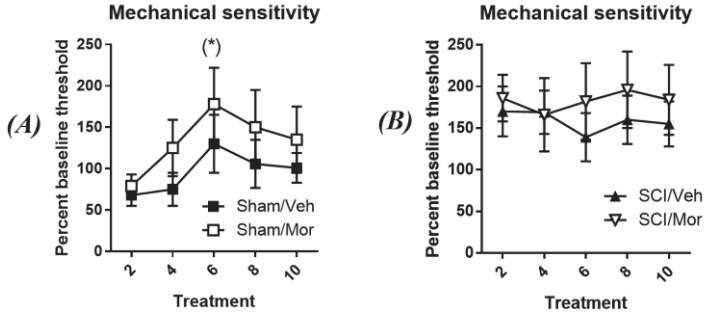
***(A)** BMS score from week 2 to week 10 were evaluated in sham and spinal cord injury (SCI) mice which were infused with saline or morphine (2.52mg/100ul) for 7 days (sham+vehicle and sham morphine groups n=10, SCI+vehicle and SCI+morphine n=9). **(B)** BMS score in sham and spinal cord injury mice which were infused with morphine (3.78mg/100ul) for 7 days (sham+vehicle and sham morphine groups n=9-12, SCI+sham and SCI+morphine n=7-9). (A and B): Two way ANOVA (* p<0.05).*

Figure 4. The effect of morphine infusion on thermal sensitivity .



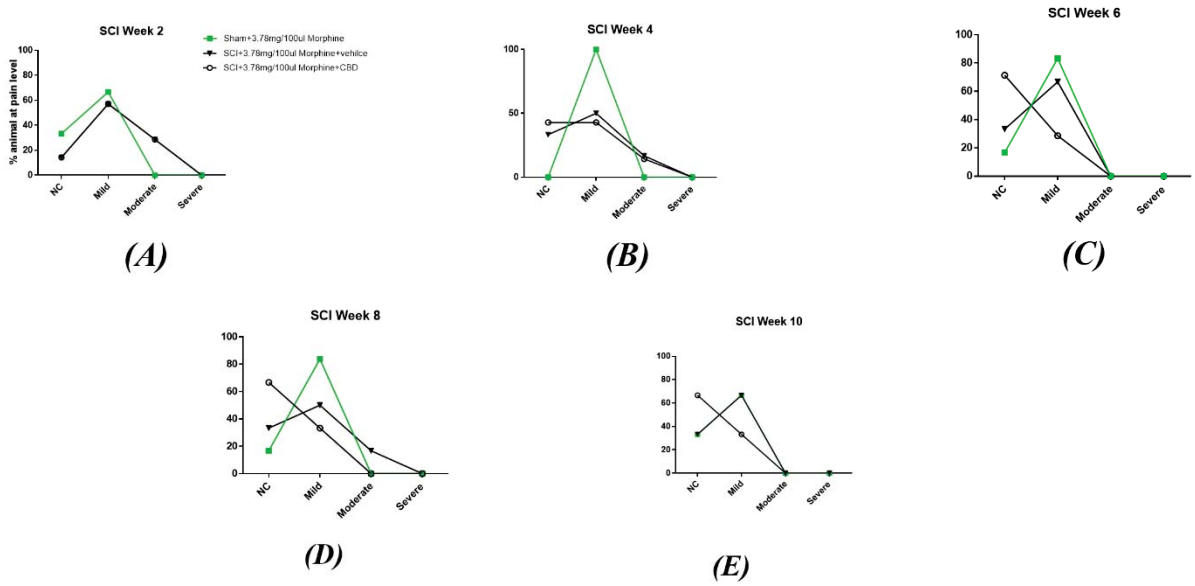
(A-E) *The sham and spinal cord injury (SCI) mice infused with morphine (2.52mg/100ul) for 7 days and thermal sensitivity were evaluated from week 2 to week 10 (sham+morphine group n=10, SCI+vehicle and SCI+morphine n=9). The time of thermal test in each mouse after injury was compared with individual baseline before surgery to get the ratio and the ratio was divided into 4 levels: <30%, 30%-60%, 60%-99%, >100%, which represented different pain degree.*

Figure 5. The effect of morphine infusion with 3.78mg/100ul mini-pump or vehicle for 7 days on mechanical sensitivity.



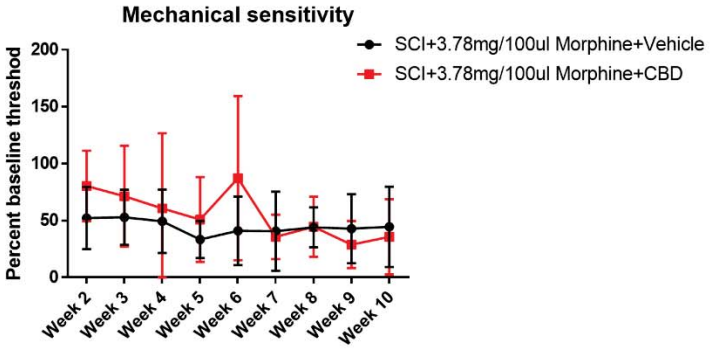
(A and B) *The sham and spinal cord injury (SCI) mice infused with saline or morphine (3.78mg/100ul) for 7 days and mechanical sensitivity were evaluated from week 2 to week 10 (sham+vehicle and sham+morphine groups n=9-12, SCI+sham and SCI+morphine n=7-9) .). (A and B): Two way ANOVA (*) p=0.07.*

Figure 6. CBD treatment ameliorated morphine's (3.78mg/100ul) effect on thermal sensitivity in spinal cord injury mice.



(A-E) *The sham and spinal cord injury mice infused with 3.78mg/100ul morphine for 7 days were treated with CBD 30mg/kg or vehicle for 7 days and the thermal sensitivity were evaluated from week 2 to week 10 (sham +morphine+ vehicle and SCI+morphine+vehicle group n=6-7, SCI+morphine +CBD n=6-8).*

Figure 7. The effect of CBD on mechanical sensitivity in spinal cord injury mice with morphine (3.78mg/100ul) infusion.



The spinal cord injury mice infused with 3.78mg/100ul morphine for 7 days were treated with CBD (30mg/kg) or vehicle for 7 days and the mechanical sensitivity were evaluated from week 2 to week 10 (SCI+morphine+vehicle group n=6-7 and SCI+morphine+CBD n=6-8) .

Conclusions:

1. Infusion with 2.52mg/100ul and 3.78mg/100ul morphine deteriorated locomotor function in spinal cord injury mice.

2. Morphine infusion with 3.78mg/100ul worsened mechanical sensation in spinal cord injury sham mice.

3. CBD treatment can ameliorate morphine's (3.78mg/100ul) effect on thermal sensation in spinal cord injury mice, but it failed to improve BMS score and bladder recovery.

Conclusion: Morphine can deteriorate the locomotor recovery and contribute to chronic neuropathic pain in spinal cord injured mice and CBD treatment can ameliorate morphine's effect on neuropathic pain.

AIM 3.

Key findings of experiments conducted under specific aim 3 are detailed below.

A.) Initial experiments were conducted to determine the pro-inflammatory effects of the Lieber DeCarli Diet, a National Institutes of Alcohol Abuse and Alcoholism-approved rodent protocol for inducing alcohol-associated liver damage, and potential anti-inflammatory effects of CBD. We determined preliminary pro-inflammatory effects of this diet using plasma borne markers. We also assessed pro-inflammatory effects of the ethanol diet on brain and liver tissue and did not find robust inflammatory effects in these tissues. We did find, however, that CBD and another non-psychoactive Cannabis constituent beta-caryophyllene may attenuate plasma markers of inflammation.

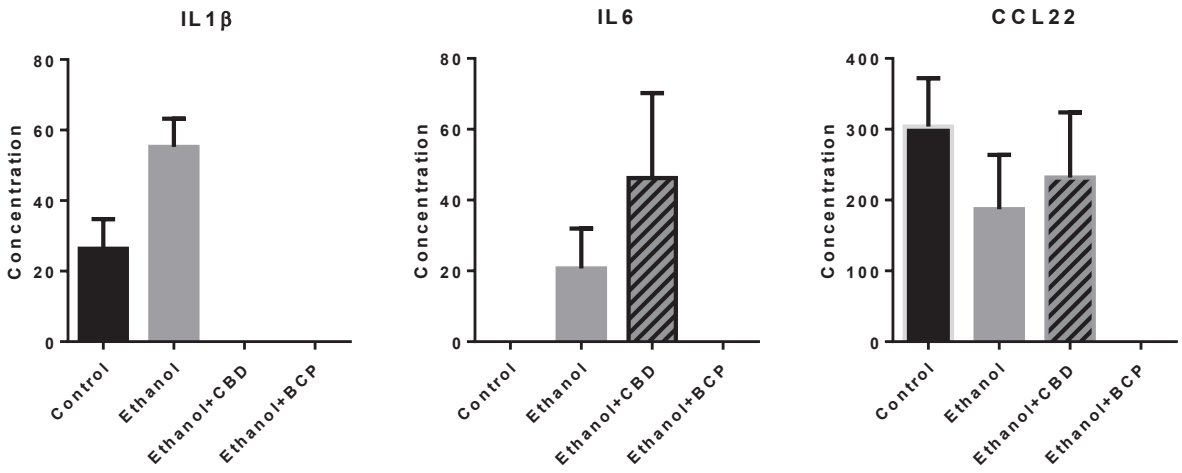


Figure 1. Ethanol intake increases the pro-inflammatory cytokines Il-1B and IL-6. CBD significantly decreased plasma IL-1B but not IL-6. The cannabinoid CB2 agonist naturally found in Cannabis, BCP, significantly decreased both as well as the pro-inflammatory cytokine CCL22.

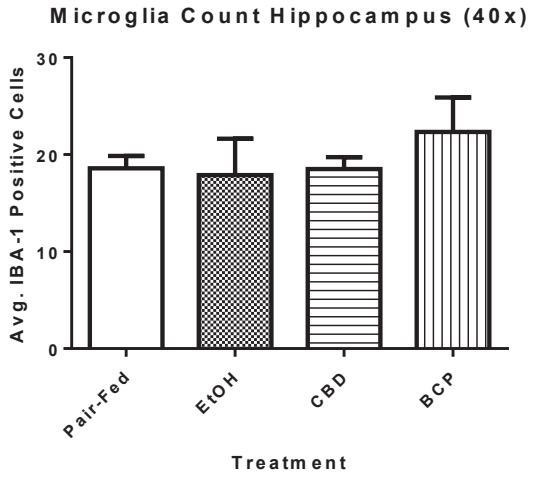


Figure 2. Ethanol consumption does not increase microglial cell number in the hippocampus.

B.) Next, experiments were conducted to determine the impact of the Leiber DeCarli Diet and SCI on locomotor recovery, development of neuropathic pain, and liquid diet consumption. We determined that ethanol consumption under this model produced a modest attenuate of locomotor recovery following SCI, but did not significantly impact development of neuropathic pain following SCI. The most striking effect was that following SCI, the control diet group showed a significant increase in food consumption while the ethanol diet group consumed significantly less of their ETOH diet. Control and ethanol diet consumption did not differ between sham (non SCI) mice.

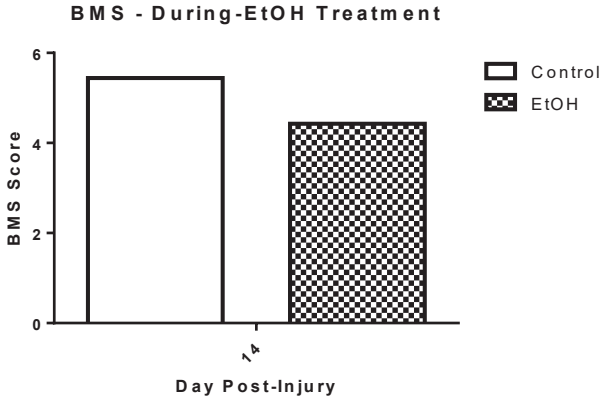


Figure 3. Ethanol consumption produces a moderate impairment of locomotor recovery following SCI as compared with control diet.

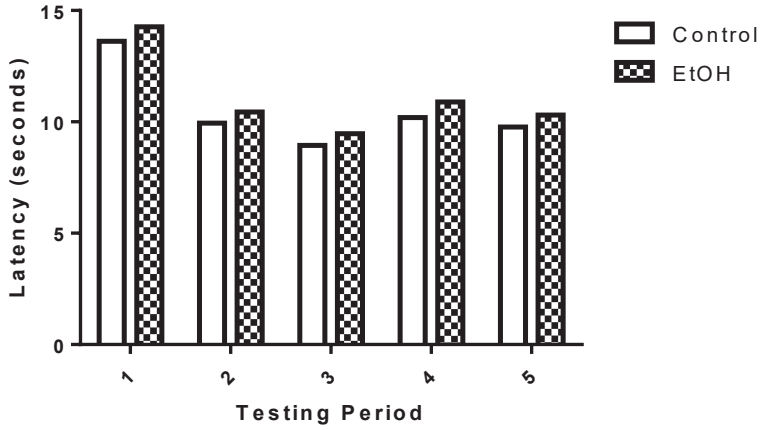


Figure 4. Latency to remove the hindpaw from a radiate heat source decreases following SCI, indicative of a development of neuropathic pain. Previous history of ethanol consumption does not exacerbate development of heat sensitivity following SCI as compared with control diet.

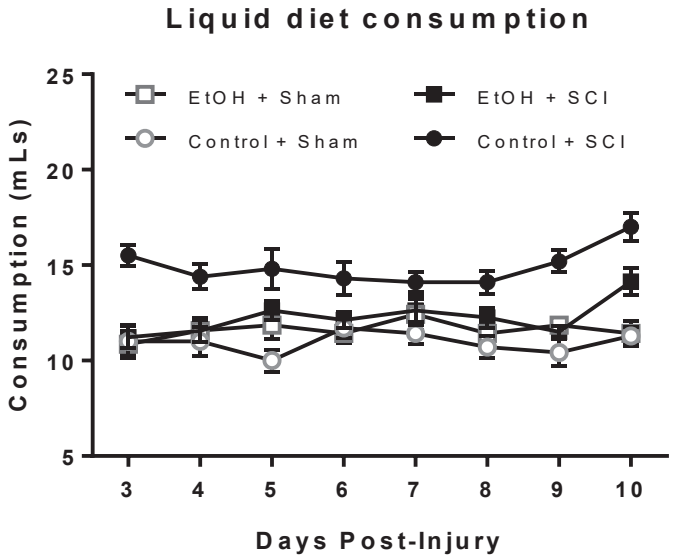


Figure 5: Four groups of mice were trained to consume control liquid diet prior to SCI. Three days following sham or SCI surgery, One sham and one SCI group were switched to the Lieber DeCarli ethanol-containing liquid diet. Both sham groups and the ETHO SCI group consumed comparable amounts of liquid diet, while the SCI group maintained on the control diet showed a significant increase in consumption. Two-way ANOVA reveals a significant effect of Treatment [$F(3, 277) = 77.59$, $P<0.0001$. Multiple comparisons analysis reveals significant differences between Control+SCI and Control Sham, and between Control+SCI and ETOH+SCI.

Conclusions: The Lieber-DeCarli ethanol diet used in the present studies produced weak pro-inflammatory effects and weak effects on SCI recovery. Future studies are necessary to further determine whether a more relevant, pro-inflammatory model of ethanol consumption would

reveal more interactions with SCI and whether this effects would be impacted by treatment with CBD.

Training and professional development: Nothing to report

Results disseminated to communities of interest: Nothing to report

Plans for the next reporting period: Final report, no plans

IMPACT

a. **Impact on the fields of cannabinoids and spinal cord injury.** Our results demonstrate that treatment with CBD can attenuate the development of pain sensitivity following SCI in a mouse model. These results provide the first evidence for a protective effect of CBD in an animal model of neuropathic pain following spinal cord injury and support ours and other's findings in other animal models of neuropathic pain. The results from these experiments also heavily implicates the ability of CBD to suppress T cell activation and recruitment in its protective action against development of central neuropathic pain. Our results also demonstrate that in SCI, morphine can exacerbate recovery following spinal cord injury and that some of these adverse effects can be improved by combining CBD treatment.
b. **Impact on other fields.** These discoveries have implications for drug discovery in that CBD may be highlighted as a novel chemical scaffold for a protective molecule against immune cell activation and CNS infiltration. These discoveries have implications for the understanding of the mechanisms of CBD in other disease models with a significant immune/inflammatory component. For example, we have recently submitted a grant to the NIH regarding the therapeutic potential of CBD for the treatment of traumatic brain injury
c. **Impact on technology transfer.** These results are likely to make an impact on commercial technology as the investigators are currently in communication with several biotechnology companies regarding patent concepts for CBD and/or synthesis of CBD analogues for distinct therapeutic targets. For example, we have recently received funding by the National Institutes on Drug Abuse to investigate the CBD analogue KLS-13019 on neuropathic pain and associated inflammation.
d. **Impact on society.** Results such as the ones presented here wherein non-psychoactive cannabinoid agents show evidence-based efficacy in models of chronic pain improve public knowledge and attitudes on the therapeutic potential of cannabinoid-based treatment strategies.

CHANGES/PROBLEMS: Nothing to report

PRODUCTS

a. **Publications, conference papers, and presentations.** Poster presentation by project-supported postdoctoral fellow Hongbo Li, PhD at annual Mid-Atlantic Pharmacology Society meeting October 2015 entitled “Effect of the non-psychoactive cannabinoid CBD on spinal cord injury neuropathic pain”
b. **Websites.** Nothing to report
c. **Technologies/techniques.** Nothing to report
d. **Inventions, patent applications, licenses.** Nothing to report
e. **Other products.** We have since progressed in our collaboration with KannaLife Sciences stemming from these and other results from our laboratory to secure funding to pursue research and development into the CBD analogue KLS-13019 which possesses among other things a vastly improved pharmacokinetic profile compared with CBD.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

e. Personnel

- **Name: Sara Jane Ward**
  Project Role: PI
  Person months worked: 3
  Contribution to project: Supervising the research program, designing the experiments, provide assistance in the laboratory when needed, analyzing the data (together with the other members of the research team), and composition of the reports and manuscripts.
- **Name: Ronald Tuma**
  Project Role: Co-investigator
  Person months worked: 2.8
  Contribution to project: Supervising the procedures directly related to spinal cord injury in mice and assisting the PI in design of experiments and interpretation of results.
- **Name: Doina Ganea**
  Project Role: Co-investigator
  Person months worked: 1.2
  Contribution to project: Supervising personnel regarding the immunology evaluations and assisting Dr. Ward in the planning of the experiments and analysis of data related to these endpoints.
- **Name: Hongbo Li**
  Project Role: Postdoctoral Fellow
  Person months worked: 9
  Contribution to project: Conducting the spinal cord injuries and behavioral assessments, as well as immunohistochemistry and analysis of data.
- **Name: Weimin Kong**
  Project Role: Research Associate
  Person months worked: 6
  Contribution to project: Conducting flow cytometry, RT-PCR, and cell culture analyses.

SPECIAL REPORTING REQUIREMENTS, AND APPENDICES. None